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Method validation and determinations of levofloxacin, metronidazole and sulfamethoxazole in an aqueous pharmaceutical, urine and blood plasma samples using quantitative nuclear magnetic resonance spectrometry

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ABSTRACT

Selective, rapid and accurate quantitative proton nuclear magnetic resonance (qHNMR) method for the determination of levofloxacin, metronidazole benzoate and sulfamethoxazole in aqueous solutions was developed and validated. The method was successfully applied to the determinations of the drugs and their admixtures in pharmaceutical, urine and plasma samples. Maleic acid and sodium malate were used as internal standards. Effect of temperature on spectral measurements was evaluated. Linear dynamic ranges of 0.50–68.00, 0.13–11.30 and 0.24–21.00 mg per 0.60 mL solution were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole, respectively. Average recovery % in the range of 96.00–104.20 ± (0.17–2.91) was obtained for drugs in pure, pharmaceutical, plasma and urine samples. Inter and intra-day analyses gave average recoveries % in the ranges 96.10–98.40 ± (1.68–2.81) and 96.00–104.20 ± (0.17–2.91), respectively. Instrumental detection limits ≤0.03 mg per 0.6 mL were obtained for the three drugs. Developed method has demonstrated high performance characteristics for analyzing investigated drugs and their admixtures.

Student *t*-test at 95% confidence level revealed insignificant bias between the real and measured contents of investigated drugs in pure, pharmaceutical, urine and plasma samples and its admixtures. Application of the statistical *F*-test revealed insignificant differences in precisions between the developed method and arbitrary selected reference methods.

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1. Introduction

Several quantitative nuclear magnetic resonance (qHNMR) methods for the determination of drugs and their metabolites have been reported in the last years. qHNMR has the advantages of being fast, non-destructive, highly selective and requires easy sample preparation without derivatization. It also gives information about the molecular structures, components in admixtures and their ratios without signals' calibrations. Low sensitivity is the most significant disadvantage of qHNMR. However, continuous improvements in the strengths of external magnetic fields and electronics of instruments, have improved the sensitivity [1]. Although qHNMR has not yet approved generally in pharmaceutical industry, it has been accepted in a number of international pharmacopoeias. Several qHNMR methods have been reported for analysis of drugs in pharmaceutical and biological samples [2,3], in vivo and in vitro medical assays [4], food analysis [5], forensic analysis of Xenobiotics in biological fluids [6], analysis of drug metabolites [7], determining residual solvents [8] and for determining isomeric composition of drugs [9].

Levofloxacin, 9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido(1,2,3-de)-1,4-benzoxazine-6-carboxylic acid, is the S (−) enantiomer of the ofloxacin racemic mixture. It is a synthetic fluorinated quinolone derivative, having activity against Gram (+) and (−) bacteria. It acts as antibiotic by inhibiting the DNA-gyrase of the bacteria [9].

Several methods have been reported for the determination of levofloxacin in pharmaceutical and biological fluids based on high performance liquid chromatography (HPLC) [10–13], adsorptive stripping voltammetry at glassy carbon electrodes [14], synchronization-first-derivative fluorescence spectroscopy [15], spectrofluorometry [16], colorimetry [17] and qHNMR [18].

Metronidazole benzoate, 2-(2-methyl-5-nitroimidazole-1-yl)ethyl benzoate, is an antiprotozoal, anti-amebic and antibacterial drug. Metronidazole is one of the most commonly used drugs all over the world, one of the top 100 most prescribed drugs in United States and one of the 10 most-used drugs during pregnancy. Metronidazole is the principal treatment for Helicobacter pylori infections, amebiasis, giardiasis, trichomoniasis and Crohn's disease. It is also extensively

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used in the treatment of bacterial vaginosis, anaerobic bacterial infections and used as prophylactic antibiotic in surgical interventions [7].

Methods based on potentiometry [19], supercritical fluid chromatography [20], HPLC [21,22], derivative spectrophotometry [23], flow injection analysis [24], spectrophotometry [25], fluorescence [26], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [27], electrochemistry [28,29], photometry [30] and qHNMR [31] were reported for the determination of metronidazole in pharmaceutical, plasma, saliva and gingival crevicular fluid.

Sulfamethoxazole, 5-methyl-3-sulfanilamidoisoxazole, is a common antibiotic that is largely used to treat respiratory diseases like pneumonia, coccidiosis, diarrhea and gastroenteritis. A combination of sulfamethoxazole and trimethoprim is used in the treatment of many infections such as urinary and respiratory tract infections. Animals are also treated by a combination of drugs containing sulfamethoxazole.

Sulfamethoxazole in presence of trimethoprim was determined in biological fluids using HPLC with UV and tandem-mass detections [32] as well as with diode array and fluorescence detections [33]. Separation of sulfamethoxazole from trimethoprim using two elution solvents and two successive chromatographic operations has been reported [34]. Analyses of sulfamethoxazole in milk [35,36], water [37], waste water [38], sewage sludge [39] and meat [40] using HPLC, have been reported. Determination of sulfamethoxazole in pharmaceuticals using capillary electrophoresis [41], qHNMR [32] and boron-doped diamond electrode [42] was also reported.

Quantifying pharmaceutical and biological compounds in their natural physiological fluids using NMR is still a challenging task due to the overwhelming strong water signal over the observed analyte's signal. Therefore, this work aims to develop a validated, accurate, precise and selective qHNMR method for the determination of the three antibiotics; levofloxacin, metronidazole benzoate and sulfamethoxazole; in aqueous pharmaceutical, urine and plasma samples. Reduction of water signal by suitable irradiation power has been done using the homonuclear gated decoupling measurement protocol supported to the Joel 300-MHz FT-NMR spectrometer. ^{1}H-NMR measurements in aqueous solution resulted in simplifying the analytical procedure by removing the most tedious step in the procedure, i.e. separation of the analyte from its dosage or biological matrix. Up to our knowledge, application of qHNMR for the determination of these compounds in aqueous solutions was not reported before. Results obtained were statistically evaluated and compared with previously reported chromatographic methods.

2. Experimental

2.1. Materials and reagents

The highest purity analytical grade compounds were used throughout. Levofloxacin, metronidazole benzoate and sulfamethoxazole (99.99%) were purchased from Sigma–Aldrich. Maleic acid, sodium malate, deuterium oxide and sodium borate were purchased from Merck.

Unibiotic, Flagyl and Sutrim pharmaceutical dosage forms were purchased from the local market. Unibiotic capsules contain 500.00 mg levofloxacin/capsule produced by HI-PHARM, Cairo, Egypt. Flagyl tablets contain 500.00 mg metronidazole benzoate/tablet produced by Alexandria Company for pharmaceuticals, Alexandria, Egypt. Sutrim tablets contain 400.00 mg sulfamethoxazole and 80.00 mg trimethoprim/tablet produced by Memphis, Cairo, Egypt.

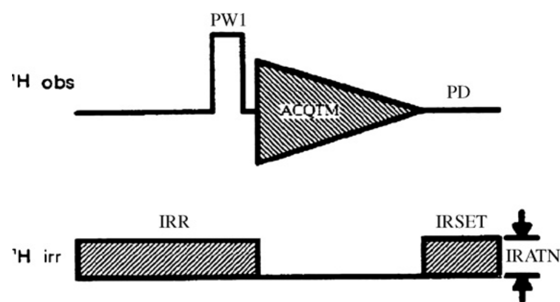


Fig. 1. Pulse sequence in ^{1}H-NMR – homonuclear gated decoupling measurement protocol (hmg). $^{1}H_{obs}$ is the observed proton, PW1 is the observation pulse, PD is the waiting time of repetition pulses, IREST is the resonance frequency of the irradiated peak, IRATN is the attenuator value for adjusting the irradiation power, $^{1}H_{irr}$ is irradiated proton and IRR is the irradiated signal.

2.2. Apparatus

All ^{1}H-NMR measurements were made using a 300-MHz FT-NMR spectrometer (JEOL-NM-LA300), Japan, supported with superconducting electromagnet cooled to cryogenic temperature using liquid helium (Oxford, GB). The magnet and coolant are put in an outer jacket cooled to 77 K with liquid nitrogen. The magnet operates at field strength of 7.1 T.

A CyberScan 510-PC pH meter, Singapore, was used for pH measurements. Dissolution and centrifugation were made using ultrasonic bath (Branson-3200, USA) and centrifuge (Beckman Coulter, USA). Evaporation and freeze drying were made using evaporator (Zymark-Turbo-Vap-LV, USA) and freeze dryer (Labconco, USA). A Labinco Vortex mixer, UK, was used for sample mixing.

2.3. ^{1}H-NMR measurement condition

All ^{1}H-NMR spectra were recorded in the chemical shift range δ^{1}H 0.0–10 ppm and referenced to TMS, $\delta = 0.0$. The NMR probe was maintained at 18 °C throughout the whole measurements. Typically, 500 free induction decays (FIDs) were collected for each sample into 32,768 data points using spectral width of 6009.6 Hz, digital resolution of 32,768/6009.6 = 5.45 points/Hz and acquisition time of 5.453 s. A relaxation delay time of 5 s was applied to ensure full T_1 relaxation between successive scans. A pulse angle of 90°, was used and line broadening factor of 0.37 Hz was applied prior to Fourier transformation. Symmetrical, well separated signals were automatically integrated. Closely spaced signals were defined manually and integrated by summing up the points within the integration range.

The homonuclear gated decoupling protocol (hmg) was used for reducing the intensity of the strong water signal and allowing the objective signal from the analyte to be detected. Measurement in hmg mode has been done using observation pulse width of 90/2, delay time of 5 s, irradiation power (IRATN) of 200, scan's number ≥ 500, receiver gain (r_g) ≥ 10, dummy pulses ≥ 4 and broadening factor (BF) ≤ 3. This condition has significantly reduced the intensity of the residual water signal shown at δ^{1}H = 4.65–4.70 ppm. NMR pulse sequence in hmg is represented in Fig. 1.

2.4. Effect of temperature

The effect of temperature change on ^{1}H-NMR spectra of levofloxacin, metronidazole benzoate and sulfamethoxazole was tested by recording the spectra of 10.0 mg of each compound dissolved in deuterated aqueous solution at 18, 25, 30, 35, 40 and 45 °C and applying the conditions described in Section 2.3. Changes in chemical shifts of corresponding signals were evaluated.

2.5. Analysis of pure samples

Stock solutions of levofloxacin (2.77×10^{-1} mol L^{-1}) and sulfamethoxazole (2.96×10^{-1} mol L^{-1}) were prepared in borate buffer, pH 10. A stock solution of metronidazole benzoate (2.29×10^{-1} mol L^{-1}) was prepared in 0.1 mol L^{-1} HCl. Volumes equivalent to 0.50–68.0 mg levofloxacin, 0.13–11.3 mg metronidazole benzoate or 0.24–21.5 mg sulfamethoxazole were transferred to NMR tubes. Accurate weights of maleic acid or sodium malate (0.17–7.5 mg) were added as internal standard. A 0.1 mL D_2O was added to each tube as field frequency lock-solvent and solutions were made up to 0.6 mL by same solvent. Solutions were thoroughly mixed on the vortex mixer, and its ^{1}H-NMR patterns were recorded using the hmg measurement protocol (Section 2.3) Spectra obtained were manually corrected for phase and baseline distortions. Signals at 8.13, 8.40, 7.42 and 5.79 ppm specific to levofloxacin, metronidazole benzoate, sulfamethoxazole and malate were integrated and used for quantifying the correspondent drug, respectively.

Calibration graphs were constructed by plotting normalized area of the selected signal with respect to the area of internal standard versus the milligram amount of the drug. Three replicates from each sample were measured.

2.6. Analysis of pharmaceutical samples

A 5–10 Unibiotic capsules, Flagyl tablets or Sutrim tablets were weighed and thoroughly ground into finely divided powders. The resultant Unibiotic or Sutrim powders (levofloxacin or sulfamethoxazole) were dissolved into 100.00 mL borate buffer solution, pH 10 whereas Flagyl powder (metronidazole) was dissolved into 100.00 mL of 0.1 mol L^{-1} HCl. Portions equivalents to 10.00–23.30 mg levofloxacin, 20.00–43.00 mg metronidazole benzoate or 4.00–10.00 mg sulfamethoxazole were accurately transferred to NMR tubes. An appropriate amount of the internal standard (1.02–8.36 mg) was added to each tube followed by 0.1 mL D_2O. Solutions were made up to 0.60 mL using the same buffer, vortexed to ensure complete mixing and its ^{1}H-NMR patterns were recorded using the hmg measurement protocol. Resulted spectra were corrected for phase and baseline distortions. Selected signals specific to levofloxacin, metronidazole benzoate, sulfamethoxazole and malate were integrated and used for quantifying correspondent drugs using the calibration graphs (Section 2.5). Three replicates from each sample were measured.

2.7. Analysis of spiked urine samples

Portions of stock pure drug solutions equivalents to 10.00–20.00 mg levofloxacin, 5.00–15.00 mg metronidazole benzoate or 5.00–15.00 mg sulfamethoxazole were transferred to NMR tubes. A 0.4 mL fresh urine was added to each tube followed by accurate weight (1.00–5.00 mg) of internal standard (maleic/malate) and 0.1 mL D_2O as solvent locker. Solutions were made up to 0.6 mL using the corresponding solvent, vortexed and its ^{1}H-NMR patterns were recorded using the hmg measurement protocol. Spectra were manually corrected for phase and baseline. Selected signals were integrated and drugs were quantified using the calibration graphs from Section 2.5. Three replicates from each sample were measured.

2.8. Analysis of spiked plasma samples

Portions of stock pure drug solutions equivalents to 5.00–15.00 mg levofloxacin, 3.75–30.00 mg metronidazole benzoate or 1.40–10.00 mg sulfamethoxazole were transferred to NMR tubes. A 0.4 mL fresh plasma was added to each tube followed by accurate weight (1.00–5.00 mg) of the internal standard (maleic/malate) and 0.1 mL D_2O. Solutions were made up to 0.6 mL, vortexed for complete dissolution and its ^{1}H-NMR patterns were recorded using the hmg measurement protocol. Spectra were corrected for phase and baseline. Selected signals were integrated and drugs were quantified using the calibration graphs from Section 2.5. Three replicates from each sample were measured.

2.9. Analysis of levofloxacin and sulfamethoxazole admixtures in pure, pharmaceutical, urine and plasma samples

Portions of stock pure drugs' solutions equivalents to 2.40–12.80 mg levofloxacin and 2.45–10.12 mg sulfamethoxazole were mixed into NMR tubes. A 2.50 mg of the internal standard and 0.1 mL of D_2O were added to each tube. Solutions were made up to 0.6 mL using borate buffer, pH 10, vortexed and its ^{1}H-NMR patterns were recorded using the hmg measurement protocol.

Using the Unibiotic and Sutrim stock solutions (Section 2.6), portions equivalents to 3.20–3.50 mg levofloxacin and 3.20–3.50 mg sulfamethoxazole were mixed into NMR tubes. A 3.11 mg internal standard and 0.1 mL D_2O were added to each tube. Solutions were made up to 0.6 mL using borate buffer, vortexed and its ^{1}H-NMR patterns were recorded using the hmg measurement protocol.

Portions of pure stock solutions equivalents to 3.15–6.30 mg levofloxacin and 3.50–5.50 mg sulfamethoxazole were mixed into NMR tubes. A 0.4 mL fresh healthy human urine or plasma was added to each tube followed by 4.0 mg internal standard and 0.1 mL D_2O. Solutions were made up 0.6 mL with borate buffer, and its ^{1}H-NMR spectra were recorded using the hmg measurement protocol. In all cases, selected signals were integrated and drugs were quantified using the calibration graphs from Section 2.5.

2.10. Analysis of administrated drugs in human urine and plasma samples

A 2000 mg of metronidazole benzoate (Flagyl), 2000 mg levofloxacin (Unibiotic) or 1600 mg sulfamethoxazole (Sutrim) was administrated by two volunteers. Volunteers were instructed to fast overnight before administration and drink a large amount of water 2 h after drugs' administration to ensure sufficient urine production during samples' collection. After drug administration, urine and plasma, samples were collected. Plasma samples were collected after 1.0 and 2.0 h in case of levofloxacin, 2.0 and 4.0 h in case of sulfamethoxazole and 1.5 and 3.0 h in case of metronidazole. Prior to drug administration, reference urine and plasma, samples were collected for comparison. Collected urine and plasma samples were protected from light and stored at −20 °C.

Prior to ^{1}H-NMR measurements, urine or plasma samples were thawed, vigorously mixed and freeze dried for 24 h. Resultant residues were centrifuged at 4000 rpm for 10.0 min. A 0.4 mL of each supernatant solution was transferred to NMR tube followed by adding an appropriate amount of the internal standard and 0.1 mL D_2O. Resulting solutions were made up to 0.6 mL using the appropriate solvents and its ^{1}H-NMR spectra were recorded using the hmg measurement protocol. Selected signals were integrated and quantified.

To apply the standard addition method, successive volumes of stock solutions equivalents to 1.83–21.80 mg levofloxacin, 3.0–20.0 mg metronidazole benzoate or 1.50–12.00 mg sulfamethoxazole were added to 0.4 mL of the above urine or plasma samples. Resulted solutions were vortexed for complete dissolutions, and its ^{1}H-NMR spectra were recorded using the hmg measurement protocol. Spectra were corrected and selected signals were integrated. Calibration graphs of normalized areas with respect to internal standard were plotted versus milligram amounts

of drugs. Linear graphs obtained were extrapolated. At least, three replicates were analyzed.

2.11. Method validation

Developed ^{1}H-NMR method was validated for its specificity, linearity, sensitivity, detection limit and quantification limits, accuracy and precisions.

3. Results and discussion

Application of qNMR for the analysis of pharmaceutical compounds in aqueous natural biological fluids is still a challenging task due to the overwhelming strong water signal over the observed signals. In our measurements, the strong water signal shown around δ^{1}H = 4.65–4.70 ppm was irradiated by radiofrequency pulses having irradiation power of ~200 W and 5.0 s delay time of repetition using the homonuclear gated decoupling measurement protocol (hmg) Application of the hmg protocol has significantly reduced the intensity of residual water signal and enabled detection of observed signals (Fig. 1).

Measurements of longitudinal relaxation times for the signals at 8.13, 8.40 and 7.42 ppm specific to levofloxacin, metronidazole benzoate and sulfamethoxazole have revealed T_1 values of 0.75, 2.10 and 0.62 s, respectively. To achieve high sensitivity, an acquisition time of 5.453 s and a relaxation delay time of 5.0 s were applied during the hmg measurements. This made a total of 10.453 s between successive scans, enough to ensure full T_1 relaxation and allow excited nuclei to re-establish their equilibrium z-magnetization after acquisition of the FID information and prior to the application of the next pulse.

3.1. Assignment of signals in the ^{1}H-NMR spectra of levofloxacin, metronidazole benzoate and sulfamethoxazole

Structural formula of levofloxacin, metronidazole benzoate and sulfamethoxazole are shown in Scheme 1 and its ^{1}H-NMR spectra are shown in Figs. 2–4. Fig. 2a shows the ^{1}H-NMR spectrum of levofloxacin in aqueous buffer solution. Signals at δ = 6.50 and 8.13 ppm are assigned to the 5-H and 2-H protons, respectively. The signal at δ = 3.50 ppm is assigned to the 2′, 3′, 5′ and 6′ protons and signals at δ = 3.20 and 2.75 ppm are assigned to the 1b and 4′a protons, respectively. The signal at δ = 4.65 ppm is attributed to residual water and the signals at δ = 6.04–5.34 are attributed to the OH. The isolated, sharp singlet signal at δ = 8.13 ppm was selected for the quantitative determination of levofloxacin. Levofloxacin's signals were up field shifted by 0.1–1.08 ppm relative to previously reported chemical shifts measured in DMSO-d_6 [18].

Fig. 3a shows the ^{1}H-NMR spectrum of metronidazole benzoate from aqueous HCl solution. The singlet at δ = 8.40 ppm is attributed to the 1-H proton, and the doublet at δ = 4.50 is attributed to the 2-H, 3-H protons. These protons were shown at δ = 8.06 and 4.68–4.77 ppm in DMSO-d_6 [31]. The singlet at δ = 2.60 ppm is assigned to the methyl protons whereas the signal at δ = 6.25 ppm is attributed to the phenyl protons. These protons were shown at 2.52 and 7.52 in DMSO-d_6 [31]. The signal at δ = 8.40 ppm was selected for quantifying metronidazole benzoate in aqueous solution.

Fig. 4a shows the ^{1}H-NMR spectrum of sulfamethoxazole. The signals at δ = 5.82 and 5.53 ppm are attributed to the 4a-H and NH_2 protons, respectively. The sharp singlet at δ = 2.02 ppm is attributed to the methyl protons on ring II and the signal at δ = 4.70 ppm is attributed to residual water. The signal appeared at δ = 6.66 ppm is attributed to 3b-H and 5b-H protons and the doublet at δ = 7.42 ppm is attributed to the 2b-H and 6b-H protons. The 7.42 ppm signal was selected for quantifying sulfamethoxazole. Metronidazole and

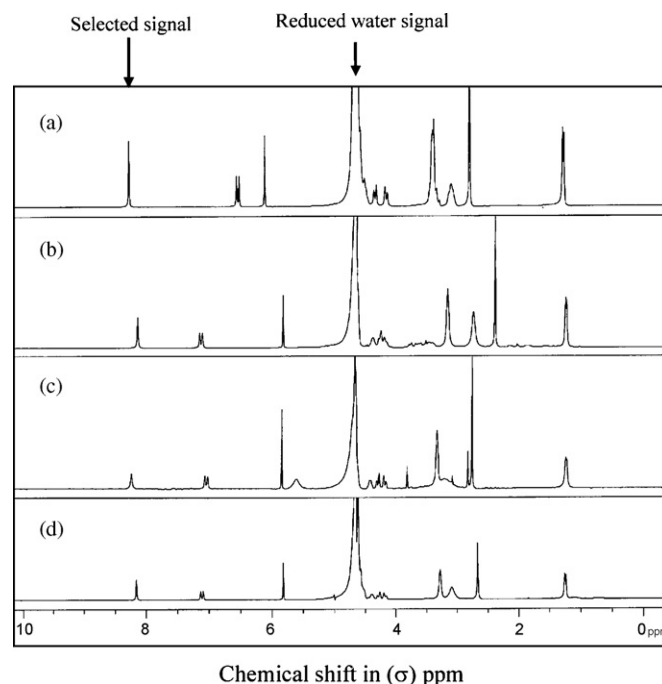


Fig. 2. ^{1}H-NMR spectra of levofloxacin collected from pure (a), pharmaceutical (b), urine (c) and plasma (d) samples. Samples were dissolved in aqueous borate buffer solution pH 10 and the hmg pulse sequence was applied to reduce the water signal.

sulfamethoxazole's signals were slightly up field shifted relative to previously reported chemical shifts measured in DMSO-d_6 [31].

^{1}H-NMR spectra of maleic acid in aqueous-D_2O solution and DMSO-d_6 showed the methylene protons as singlet at δ = 5.79 ppm [18,31]. This signal was used as a standard signal for ^{1}H-NMR quantification.

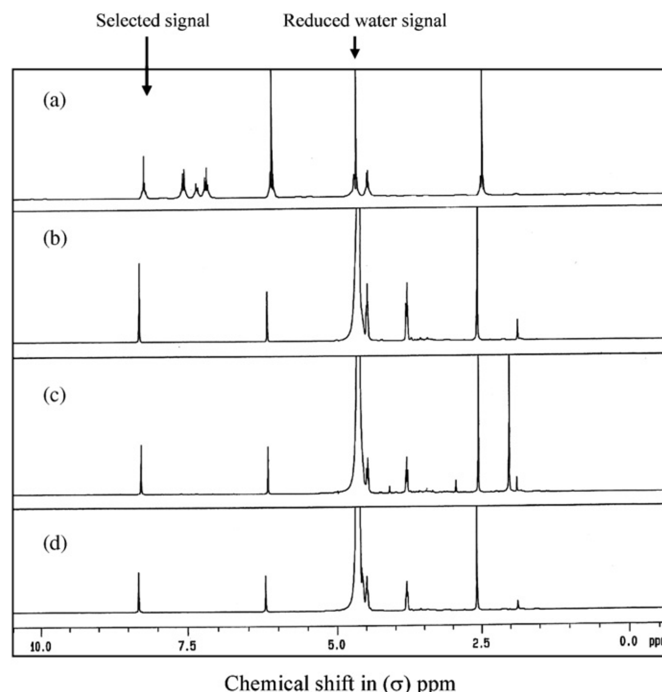
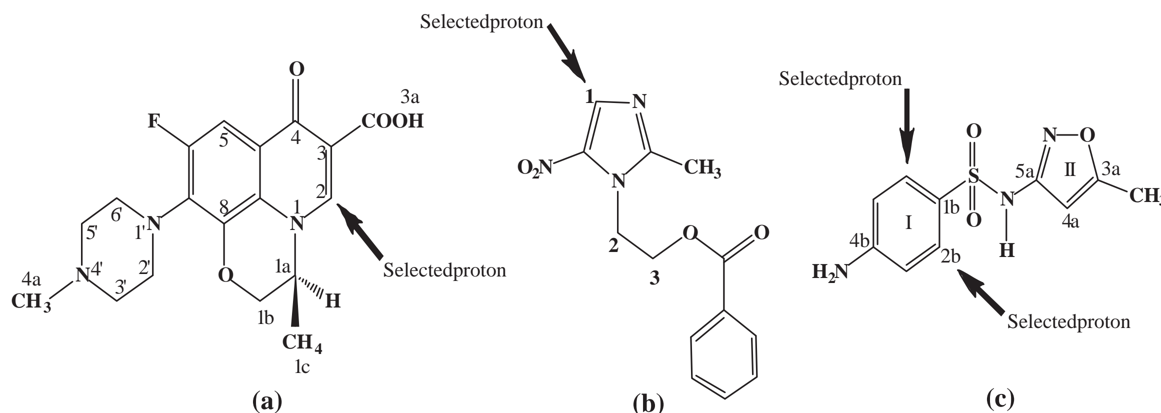


Fig. 3. ^{1}H-NMR spectra of metronidazole benzoate collected from pure (a), pharmaceutical (b), urine (c) and plasma (d) samples. Samples were dissolved in 0.1 mol L^{-1} M HCl solution and the hmg pulse sequence was applied to reduce the water signal.



Scheme 1. Schematic representation of structural formula of levofloxacin (a), metronidazole benzoate (b), sulfamethoxazole (c).

^{1}H-NMR signals are generally characterized by its chemical shifts, multiplicity, line width and relative intensity. Spectra collected from aqueous solutions have shown slight changes in chemical shifts of some signals compared to spectra collected from DMSO-d_6. The reason could be attributed to proton's chemical exchange occurs in aqueous acidic and alkaline solutions. Protons' shielding by urine and plasma matrices could also contribute to these shifts (Figs. 2–4a, b, c and d) [43].

3.2. Effect of temperature

Change in probe temperature during ^{1}H-NMR measurement can affect signal separation, extent of self-association and reliability of collected results [44]. Table 1 shows the effect of temperature on chemical shifts of the selected signals of the three investigated drugs. Signals at δ = 8.13, 8.40 and 7.42 ppm attributed to levofloxacin-2-H, metronidazole-1-H and sulfamethoxazole-2b-H, 6b-H protons were found sensitive to temperature changes in the range 291–318 K. Increasing the temperature from 291 to 318 K was found to shift the three signals downfield by 0.398, 0.336 and 0.224 ppm, respectively.

Changes in the chemical shifts by increasing temperature could be possibly attributed to changes in electrons' delocalization, electrons' interactions and/or to conformational inter-conversions caused by interruption of hydrogen bonding [43].

To improve accuracy and precision in our measurements, all our ^{1}H-NMR measurements were performed at 18 °C.

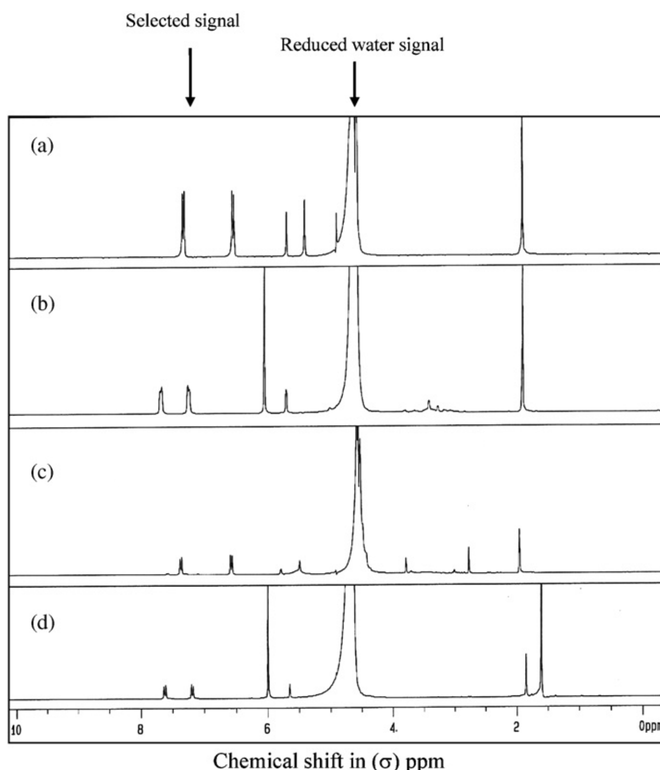


Fig. 4. ^{1}H-NMR spectra of lsulfamethoxazole collected from pure (a), pharmaceutical (b), urine (c) and plasma (d) samples. Samples were dissolved in aqueous borate buffer solution pH 10 and the hmg pulse sequence was applied to reduce the water signal.

3.3. Method validation

3.3.1. Calibration graphs and linearity

Linearity of developed method was verified by constructing the calibration graphs using the calibration curve method and the standard addition method. In the calibration curve method, different amounts of pure drugs were dissolved in aqueous borate or HCl solutions as described in Section 2.5. Calibration graphs were obtained by plotting the normalized areas of the signals at 8.13, 8.40 and 7.42 ppm with respect to the internal standard signal area at 6.20 ppm according to Eq. (1).

$$\frac{A_{drug}}{A_{int.\ stand}} \times \frac{E_{drug}}{E_{int.\ stand}} \times W_{int.\ stand} = W_{drug} \qquad (1)$$

where A_{drug} and $A_{int.\ stand}$ are the areas of selected signals of drug and internal standard, E_{drug} and $E_{int.\ stand}$ are the formula weights of drug and internal standard, $W_{int.\ stand}$ and W_{drug} are the milligram amounts of the drug and internal standard, respectively.

Determinations of drugs spiked in aqueous borate or HCl solutions gave linear dynamic ranges of 0.50–68.0, 0.13–11.30 and 0.24–21.50 mg with regression equations of $Y = 0.16X - 0.07$,

Table 1
Effect of temperature on chemical shifts of levofloxacin, metronidazole benzoate and sulfamethoxazole. Spectra were recorded using 10.0 mg drug dissolved in 0.6 mL deuterated borate buffer or 0.1 mol L^{-1} HCl.

Temperature (°C)	Chemical shift (ppm)		
	Levofloxacin	Metronidazole benzoate	Sulfamethoxazole
18	7.998	8.105	7.424
25	8.195	8.314	7.454
30	8.251	8.341	7.491
35	8.300	8.380	7.530
40	8.348	8.409	7.595
45	8.396	8.441	7.648

Table 2
Quantitative ^{1}H-NMR analysis of levofloxacin in pure, pharmaceutical, urine and plasma samples. Drug was spiked into 0.6 mL of aqueous buffer, urine or plasma solutions. Results are evaluated using the statistical Student *t* and *F*-tests.

Taken (mg)	Found[a] (mg)	Average recovery %	RSD	Bias%[b]	*t*-Value[c]	*F*-Value[d]	Ref.
Authentic							
0.50	0.51	102.00	1.51	2.00	1.73	2.28	[45]
5.20	5.05	97.20	1.29	−2.80	2.81	1.65	[45]
15.60	15.30	98.10	1.50	−1.90	2.22	2.25	[45]
26.00	25.20	97.00	1.13	−3.00	3.46	1.28	[45]
39.80	38.90	97.80	1.25	−2.20	2.31	1.57	[45]
53.50	52.00	97.20	1.58	−2.80	2.18	2.50	[45]
68.00	68.20	100.30	1.63	0.30	0.21	2.66	[45]
Tablets (Unibiotic, 500 mg)							
10.00	9.70	97.00	1.31	−3.00	2.60	2.32	[16]
12.00	11.70	97.30	1.29	−2.70	2.31	2.25	[16]
23.30	22.80	97.70	1.18	−2.30	2.08	1.90	[16]
Levofloxacin spiked in urine							
10.00	9.73	97.30	0.17	−2.70	2.75	1.31	[16]
13.00	12.60	96.80	0.26	−3.20	2.73	1.07	[16]
20.00	19.20	96.00	0.46	−4.00	2.66	1.45	[16]
Levofloxacin spiked in plasma							
5.00	4.91	98.20	1.63	−1.80	1.99	1.36	[16]
10.00	9.73	97.30	2.26	−2.70	2.13	1.42	[16]
15.00	14.70	97.70	1.98	−2.30	2.08	1.09	[16]

[a] Average of at least three determinations.
[b] $\text{Bias\%} = ((\bar{X} - X_t) \times 100)/X_t$.
[c] $\text{Student-}t = ((\bar{X} - X_t) \times \sqrt{N})/\text{SD},\ N = 3$.
[d] $F = \text{SD}_1^2/\text{SD}_2^2$, SD_1 and SD_2 are the standard deviations of developed qHNMR and reference methods, irrespectively [16,45].

$Y = 0.33X - 0.01$ and $Y = 0.23X - 0.01$ for levofloxacin, metronidazole benzoate and sulfamethoxazole, respectively. Y is the normalized area of selected signal with respect to the area of the internal standard's signal and X is the amount of drug in mg. Correlation coefficients in the range 0.994–1.000 were respectively obtained (Fig. 5a and Tables 2–4). Average recovery % of 97.00–102.00 ± (1.13–1.63), 96.20–98.50 ± (1.94–2.91) and 97.50–104.20 ± (1.54–2.42) were respectively obtained. The lower limits of calibration of 0.50, 0.13 and 0.24 mg per 0.6 mL were imposed by the sensitivity of the NMR spectrometer towards the detections of levofloxacin, metronidazole benzoate and sulfamethoxazole. The upper limits of calibration (68.0, 11.3 and 21.0 mg) were imposed by the saturation limits of the drugs into the 0.6 mL solutions.

Levofloxacin, metronidazole benzoate and sulfamethoxazole spiked in human urine and plasma samples were analyzed using the above calibration curves. Average recovery % of 96.00–97.30 ± (0.17–0.46), 100.60–101.20 ± (1.68–2.05) and 98.60–100.00 ± (1.20–1.35) were obtained for drugs spiked in urine whereas average recovery % of 97.30–98.20 ± (1.63–2.26),

Table 3
Quantitative ^{1}H-NMR analysis of metronidazole benzoate in pure, pharmaceutical, urine and plasma samples. Drug was spiked into 0.6 mL of aqueous buffer, urine or plasma solutions. Results are evaluated using the statistical Student *t* and *F*-tests.

Taken (mg)	Found[a] (mg)	Average recovery %	RSD	Bias%[b]	*t*-Value[c]	*F*-Value[d]	Ref.
Authentic							
0.13	0.13	96.20	2.91	−3.80	6.06	2.75	[21]
0.28	0.27	96.40	1.95	−3.60	3.46	1.47	[21]
0.57	0.56	98.20	1.99	−1.20	1.73	1.41	[21]
1.13	1.10	97.30	2.91	−2.70	2.47	1.52	[21]
2.26	2.22	98.20	2.55	−1.80	1.73	1.17	[21]
4.53	4.43	97.80	2.36	−2.20	2.17	1.00	[21]
6.79	6.62	97.50	2.94	−2.50	2.94	1.55	[21]
11.30	11.13	98.50	2.79	−1.50	1.84	1.40	[21]
Tablets (Flagyl, 500 mg)							
20.00	19.90	99.50	1.11	−0.50	0.79	2.21	[21]
30.00	29.14	97.10	2.03	−2.90	2.58	1.51	[21]
43.00	41.80	97.20	1.67	−2.80	2.97	1.02	[21]
Metronidazole spiked in urine							
5.00	5.04	100.80	1.89	0.80	0.77	2.04	[50]
10.00	10.12	101.20	1.68	1.20	1.48	2.58	[50]
15.00	15.09	100.60	2.05	0.60	0.92	1.74	[50]
Metronidazole spiked in plasma							
3.75	3.64	97.10	1.59	−2.90	2.72	2.09	[52]
15.00	14.80	98.70	1.01	−1.30	1.85	1.19	[52]
30.00	30.30	101.00	0.99	1.00	1.56	1.24	[52]

[a] Average of at least three determinations.
[b] $\text{Bias\%} = ((\bar{X} - X_t) \times 100)/X_t$.
[c] $\text{Student-}t = ((\bar{X} - X_t) \times \sqrt{N})/\text{SD},\ N = 3$.
[d] $F = \text{SD}_1^2/\text{SD}_2^2$, SD_1 and SD_2 are the standard deviations of developed qHNMR and reference methods, irrespectively [21,50,52].

Table 4
Quantitative ^{1}H-NMR analysis of sulfamethoxazole in pure, pharmaceutical, urine and plasma samples. Drug was spiked into 0.6 mL of aqueous buffer, urine or plasma solutions. Results are evaluated using the statistical Student *t* and *F*-tests.

Taken (mg)	Found[a] (mg)	Average recovery %	RSD	Bias%[b]	*t*-Value[c]	*F*-Value[d]	Ref.
Authentic							
0.24	0.25	104.20	2.30	4.20	2.47	2.35	[46]
1.22	1.19	97.50	2.42	−2.50	1.73	2.60	[46]
6.12	5.98	97.70	2.01	−2.30	2.02	1.80	[46]
10.50	10.30	98.10	1.59	−1.90	1.66	1.13	[46]
16.00	15.60	97.50	1.54	−2.50	3.03	1.05	[46]
21.00	20.70	98.60	1.55	−1.40	1.68	1.07	[46]
Tablets (Sutrim, 400 mg)							
4.00	3.89	97.30	1.59	−2.70	3.98	2.57	[42]
8.00	7.98	99.80	1.65	−0.20	0.29	2.39	[42]
10.00	9.95	99.50	1.81	−0.50	0.68	1.99	[42]
Sulfamethoxazole spiked in urine							
5.00	4.93	98.60	1.62	−1.40	1.52	1.38	[51]
10.00	10.00	100.00	1.20	0.00	0.43	1.32	[51]
15.00	14.80	98.90	1.35	−1.10	1.39	1.05	[51]
Sulfamethoxazole spiked in plasma							
1.40	1.37	97.90	1.75	−2.10	2.60	5.22	[53]
6.00	5.92	98.70	1.92	−1.30	1.54	5.25	[53]
10.00	9.88	98.80	2.10	−1.20	1.86	4.39	[53]

[a] Average of at least three determinations.
[b] $\text{Bias\%} = ((\bar{X} - X_t) \times 100)/X_t$.
[c] $\text{Student-}t = ((\bar{X} - X_t) \times \sqrt{N})/\text{SD},\ N = 3$.
[d] $F = \text{SD}_1^2/\text{SD}_2^2$, SD_1 and SD_2 are the standard deviations of developed qHNMR and reference methods, irrespectively [42,46,51,53].

97.10–101.00 ± (0.99–1.59) and 97.90–98.80 ± (1.75–2.10) were respectively obtained for drugs spiked in plasma (Tables 2–4).

Fig. 5b and c shows the standard addition calibration graphs for levofloxacin, metronidazole benzoate and sulfamethoxazole spiked in urine and plasma from healthy volunteers after drugs' administration. In urine, linear graphs with regression equations of $Y = 0.17X + 0.21$, $Y = 0.39X + 0.25$ and $Y = 0.22X + 0.12$ were respectively obtained with correlation coefficients of 0.999–1.000 (Fig. 5b). In plasma, linear graphs having regression equations of $Y = 0.16X + 0.05$, $Y = 0.35X + 0.17$ and $Y = 0.23X + 0.01$ and correlation coefficients of 0.998–0.999, were respectively obtained (Fig. 5c).

Application of statistical Student *t*-test to analytical results revealed *t*-values ≤ 3.46 indicating insignificant bias between measured and real contents at 95% confidence level (Tables 2–4). The statistical *F*-test indicated insignificant difference in precisions (*F*-values ≤ 2.745) between results obtained by the developed ^{1}H-NMR method and arbitrary selected HPLC method reported for analyzing levofloxacin, metronidazole benzoate and sulfamethoxazole in authentic, pharmaceutical, urine and plasma samples (Tables 2–4) [21,45,46].

3.3.2. Selectivity

Selectivity implies the ability to unequivocally assess the analyte of interest in the presence of other components. In our measurements, ^{1}H-NMR signals at 8.13, 8.40 and 7.42 ppm specific to levofloxacin, metronidazole benzoate and sulfamethoxazole were unambiguously identified in different matrices. Interference from internal standard or drugs in admixtures was not observed. However, slight change in chemical shifts of drugs' specific signals was observed in urine and plasma fluids (Figs. 2–4 and Tables 2–5).

3.3.3. Sensitivity

Sensitivity of the developed method was evaluated by estimating analytical sensitivity at the least detected amounts of investigated drugs. Analytical sensitivities of 16.00, 66.00 and 40.91 were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole, respectively. These values indicated high sensitivity for the developed method (Tables 2–4).

3.3.4. Detection and quantification limits

Method detection limit (LOD) defined as three times the standard deviation of the least determined concentration divided by the slope of the calibration curve and method quantification limit (LOQ) defined as ten times the standard deviation of the least determined concentration divided by the slope of the calibration curve, were calculated. LOD values of 0.134, 0.045 and 0.064 and LOQ values of 0.446, 0.114 and 0.213 mg per 0.6 mL were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole dissolved in aqueous borate or HCl solutions, respectively [18,31,47].

Using the standard addition calibration curves (Fig. 5b and c), LOD values of 0.015, 0.073 and 0.110 and LOQ values of 0.05, 0.245 and 0.366 mg per 0.6 mL solutions were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole in urine, respectively. In plasma, LOD values of 0.153, 0.069 and 0.114 and LOQ values of 0.50, 0.230 and 0.380 mg per 0.6 mL solutions were obtained, respectively.

Instrument detection limits (IDL) are defined as the drug concentration required to produce a signal to noise ratio of 5 (S/N = 5). IDL values of 0.022, 0.018, and 0.020 mg per 0.6 mL were respectively obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole in aqueous buffer solutions.

3.3.5. Accuracy

Relative errors % of ±(0.00–4.17) were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole in pure, pharmaceutical, urine and plasma samples (Tables 2–4). Having less than 5% error is quite enough to use the developed qHNMR method for the determination of investigated drugs in different matrices. Such high accuracy could be attributed to the accurate sample's preparation, application of hmg measurement protocol to enhance the intensity of resolution and manual phasing with a vertical expansion of peaks [48].

3.3.6. Precision

Precision in developed qHNMR is dependent on the noise level of the spectrum, line shape, quality of shimming as well as phase and baseline corrections [49]. Controlling these parameters in our measurements has revealed average relative standard deviations

Table 5
Quantitative ^{1}H-NMR analysis of levofloxacin and sulfamethoxazole admixtures in pure, pharmaceutical, urine and plasma samples. Drugs were spiked into 0.6 mL of aqueous buffer, urine or plasma solutions. Results are evaluated using the statistical Student *t* and *F*-tests.

Taken (mg)	Found[a] (mg)	Average recovery %	RSD	Bias%[b]	*t*-Value[c]	*F*-Value[d]	Ref.
Levofloxacin							
(A) Authentic							
2.40	2.40	100.00	1.68	0.00	0.00	2.82	[45]
12.80	12.90	100.80	1.43	0.80	0.94	2.05	[45]
(B) Pharmaceuticals (Unibiotic, 500 mg)							
3.20	3.16	98.80	1.86	−1.20	1.18	3.200	[16]
(C) Urine samples							
6.30	6.45	102.40	1.85	2.40	2.18	1.17	[16]
(D) Plasma samples							
3.15	3.20	101.60	2.81	1.60	0.96	2.47	[53]
Sulfamethoxazole							
(A) Authentic							
2.45	2.42	98.80	2.07	1.20	−1.04	1.90	[21]
10.12	10.15	100.30	1.28	0.30	0.40	1.37	[21]
(B) Pharmaceuticals (Sutrim, 400 mg)							
3.50	3.48	99.40	2.01	−0.60	−0.50	1.61	[21]
(C) Urine samples							
5.50	5.62	102.20	1.96	2.20	1.89	2.02	[50]
(D) Plasma samples							
3.20	3.251	101.59	2.61	1.59	1.05	2.35	[52]

[a] Average of at least three determinations.
[b] Bias% = $((\bar{X} - X_t) \times 100)/X_t$.
[c] Student-$t = ((\bar{X} - X_t) \times \sqrt{N})/\text{SD}$, $N = 3$.
[d] $F = \text{SD}_1^2/\text{SD}_2^2$, SD_1 and SD_2 are the standard deviations of developed qHNMR and reference methods, irrespectively [16,21,45,50,52,53].

RSD of 0.17–2.91% ($n \geq 3$) for levofloxacin, metronidazole benzoate and sulfamethoxazole in pure, pharmaceutical, urine and plasma samples (Tables 2–4). These values demonstrated a highly precise developed method that can be used for the determination of investigated drugs in different matrices (Table 2).

3.3.7. Reproducibility

Reproducibility was assessed by intra-day and inter-day assays. Tables 2–7 show the average relative standard deviations for intra-day and inter-day analyzed samples. RSD values in the range 0.17–2.91% and 1.30–2.81 ($n \geq 3$) were obtained for the three drugs in pure, pharmaceutical, urine and plasma samples. These results confirm high reproducibility and suggest high confidence in the use of ^{1}H-NMR in the determination of for levofloxacin, metronidazole benzoate and sulfamethoxazole in different matrices.

The above mentioned values regarding linearity, accuracy, precision and reproducibility are fairly good figures of merit for the developed qHNMR method and allow its use for determining levofloxacin, metronidazole, sulfamethoxazole and its admixtures in pharmaceutical and biological fluid samples.

3.4. Analysis of drugs in pharmaceutical samples

Determinations of levofloxacin (10.00–23.30 mg), metronidazole benzoate (20.00–43.00 mg) and sulfamethoxazole (4.00–10.00 mg) in Unibiotic, Flagyl and Sutrim dosage forms, respectively using the developed qHNMR method are shown in Tables 2–4. Average recovery % of (97.00–97.70) ± 0.18, (97.10–99.50) ± 0.50 and (97.30–99.80) ± 0.13 were respectively obtained. These results were statistically evaluated using Student *t*-test. *t*-Values of ≤2.60, ≤2.97 and ≤3.98 were respectively obtained indicating insignificant bias between the measured and real contents at 95% confidence level. Application of the statistical *F*-test revealed insignificant differences in precisions (*F*-values ≤ 2.57) between the developed qHNMR method and the arbitrary selected reference methods reported for analyzing levofloxacin, metronidazole benzoate and sulfamethoxazole in pharmaceutical dosage forms [16,21,42].

3.5. Analysis of drugs in spiked urine samples

Urine samples spiked by 10.00–20.00 mg levofloxacin, 5.00–15.00 mg metronidazole benzoate or 5.00–15.00 mg sulfamethoxazole were measured using the developed qHNMR method (Section 2.7). Average recovery % of (96.00–97.30) ± 0.30, (100.60–101.20) ± 0.19 and (98.60–100.00) ± 0.13 were respectively obtained (Tables 2–4). Application of the Student *t*-tests revealed *t*-values ≤2.70, ≤1.48 and ≤1.52 for levofloxacin, metronidazole benzoate and sulfamethoxazole, respectively. These values indicated insignificant bias between measured and real contents at 95% confidence level. Statistical *F*-test revealed insignificant differences in precisions between the qHNMR method and the arbitrary selected reference methods [16,50,51].

3.6. Analysis of drugs in spiked plasma samples

Analyses of levofloxacin (5.00–15.0 mg), metronidazole benzoate (3.75–30.0 mg) and sulfamethoxazole (1.40–10.0 mg) in spiked plasma samples using our developed qHNMR method are shown in Tables 2-4. Average recoveries % of (97.30–98.20) ± 0.28, (97.18–101.08) ± 0.17 and (97.90–98.80) ± 0.12 were respectively obtained. Student *t*-test indicated insignificant bias (*t*-values ≤ 2.72) between the measured and real contents of drugs. *F*-test indicated insignificant difference in precisions (*F*-values ≤ 2.089) between our qHNMR method and the arbitrary used reference methods [16,52,53].

3.7. Analysis of drugs' admixtures in pure, pharmaceutical, urine and plasma samples

Levofloxacin and sulfamethoxazole in admixtures prepared from pure, pharmaceutical, urine and plasma samples were determined using the developed qHNMR method (Section 2.9). Pure

Table 6
Inter-day analyses of levofloxacine, metronidazole benzoate and sulfamethoxazole in pure, pharmaceutical, urine and plasma samples. Drugs were spiked into 0.6 mL of aqueous buffer, urine or plasma solutions.

Drug	Taken (mg)	Found[a] (mg)	Average recovery %	RSD	Bias%[b]
Authentic					
Levofloxacin	26.00	25.20	96.90	1.75	−3.10
Metronidazole benzoate	11.30	11.00	97.60	1.72	−2.40
Sulfamethoxazole	10.50	10.26	97.70	2.24	−2.30
Urine					
Levofloxacin	10.00	9.71	97.10	2.06	−2.90
Metronidazole benzoate	10.00	9.85	98.50	1.73	−1.50
Sulfamethoxazole	10.00	9.81	98.10	1.94	−1.90
Plasma					
Levofloxacin	10.00	9.61	96.10	2.81	−3.90
Metronidazole benzoate	15.00	14.60	97.30	2.06	−2.70
Sulfamethoxazole	10.00	9.70	97.00	1.96	−3.00
Pharmaceuticals					
Levofloxacin	10.00	9.70	97.00	2.06	−3.00
Metronidazole benzoate	15.00	14.60	97.30	2.19	−2.70
Sulfamethoxazole	10.00	9.84	98.40	1.98	−1.60

[a] Average of at least three determinations.
[b] Bias% = $((\bar{X} - X_t) \times 100)/X_t$.

Table 7
Inter-day analyses of sulfamethoxazole in its admixtures with levofloxacin in pure, pharmaceutical, urine and plasma samples. Drugs were spiked into 0.6 mL of aqueous buffer, urine or plasma solutions.

Matrix	Taken (mg)	Found[a] (mg)	Average recovery %	RSD	Bias[b]
Authentic	10.12	9.97	98.50	1.30	−1.50
Pharmaceuticals	3.50	3.44	98.30	1.45	−1.70
Urine	5.50	5.59	101.60	1.79	1.60
Plasma	5.50	5.46	99.30	2.38	−0.70

[a] Average of at least three determinations.
[b] Bias% = $[100 \times (\bar{X} - X_t)/X_t]$.

admixtures of levofloxacin (2.40–12.80 mg) and sulfamethoxazole (2.45–10.12 mg) gave average recovery % of 100.40 and 99.60, respectively (Tables 5 and 7). A Unibiotic and Sutrim admixture having a 3.20 mg levofloxacin and a 3.50 mg sulfamethoxazole gave average recovery % of 98.80 and 99.40, respectively. Admixtures of both drugs in fresh human urine and plasma gave average recovery % of 101.60–102.40 and 100.00–102.20 for the two drugs, respectively (Tables 5 and 7).

Average relative standard deviations ≤2.81 and relative errors ≤2.38 were obtained for the determinations of the two drugs in admixtures indicating high accuracy and precision. Applying the statistical Student *t*-test and *F*-test revealed insignificant bias relative to the real contents and insignificant difference in precisions between the developed methods and the arbitrary selected reference methods (Tables 5 and 7) [16,21,45,50,52,53].

3.8. Analysis of drugs in urine and plasma samples after its administration to human

Orally administrated doses of levofloxacin, metronidazole and sulfamethoxazole are excreted as unchanged in urine in the ratios 80%, 20% and 30%, respectively. Meanwhile, a mean plasma concentration of 2.00–3.00 $\mu g\,mL^{-1}$ levofloxacin was reported after 2–3 h of an oral dose of 200 mg. Oral administrations of 250.00 mg, 500.00 mg, or 2000.00 mg of metronidazole produce peak plasma concentrations of 6.00 $\mu g\,mL^{-1}$, 12.00 $\mu g\,mL^{-1}$ or 40.00 $\mu g\,mL^{-1}$, respectively. Administration of 800.00 mg sulfamethoxazole results in plasma concentration of 57.4–68.0 $\mu g\,mL^{-1}$.

These values encouraged us to screen the amounts of unchanged drugs in human urine and plasma using our developed qHNMR method. Urine and plasma samples collected from healthy volunteers after oral administration of 1000.00 mg levofloxacin, 1000.00 mg metronidazole benzoate or 800.00 mg sulfamethoxazole were tested using the experimental conditions described in Section 2.10.

Using the calibration curve method (Fig. 5a), average concentrations of 0.66, 0.45 and 0.40 mg per 0.6 mL of solution were respectively obtained for levofloxacin, metronidazole and sulfamethoxazole in urine. Corresponding plasma concentrations ≤ 0.45 mg/0.6 mL were obtained for the same drugs (Table 8).

Application of the standard addition calibration method (Fig. 5b and c), gave average concentrations in the range of 0.60–1.10 and 0.30–0.60 mg per 0.6 mL for the three investigated drugs in urine and plasma samples (Table 8). These averages are 35.00–55.00% higher than the averages obtained using the calibration curve method. The reason could be attributed to a matrix effect encountered between the calibration curve and the standard additions method. Results obtained also confirm the possibility of using the developed qHNMR method for determining the three drugs in biological fluids as well as for quality control in pharmaceutical samples.

Table 8
Average concentrations of levofloxacine, metronidazole benzoate and sulfamethoxazole determined in human urine and plasma samples from two volunteers ($n = 3$). Oral doses of 1000 mg levofloxacin, 1000 mg metronidazole benzoate or 800 mg sulfamethoxazole were orally administrated before samples' collections. Results were calculated using the calibration curve and the standard addition methods.

Drug	Found in urine (mg)		Found in plasma (mg)	
	Calib. Curve	STD addition	Calib. Curve	STD addition
Levofloxacin	0.66	1.10	ND	0.30
Metronidazole benzoate	0.45	0.80	0.45	0.60
Sulfamethoxazole	0.40	0.60	ND	0.45

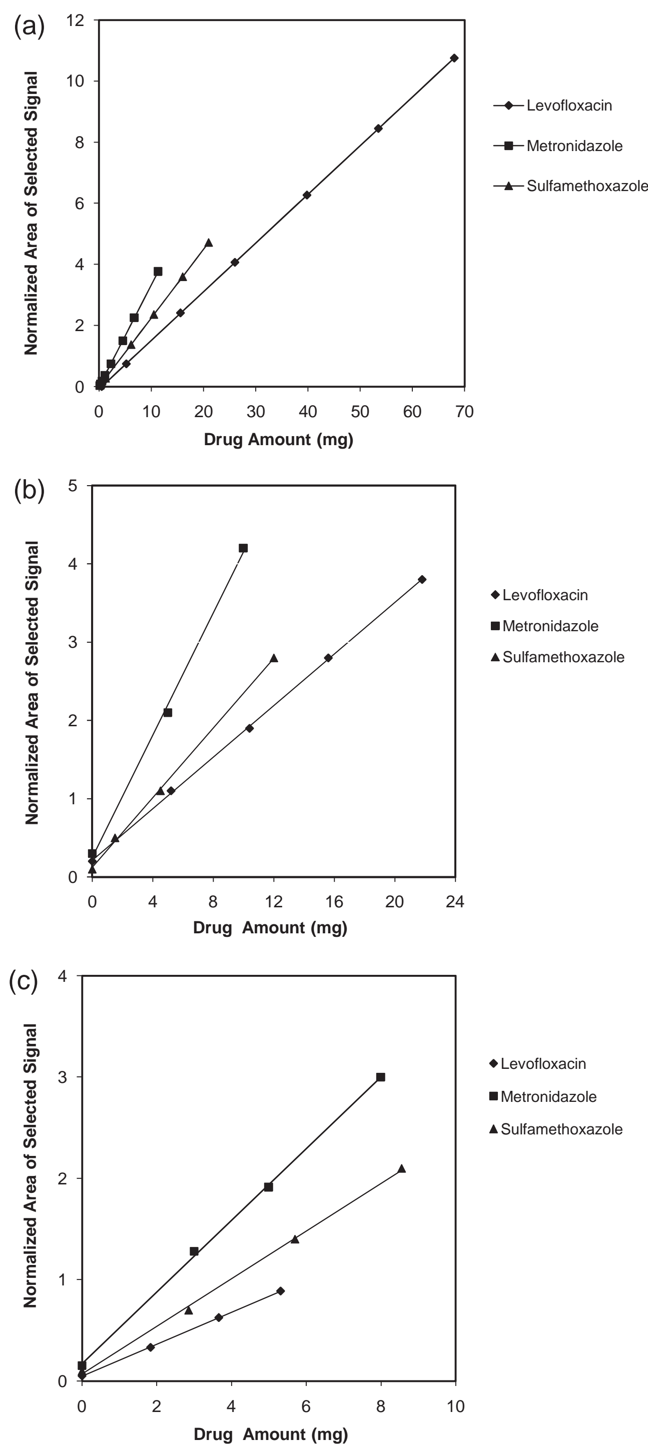


Fig. 5. Calibration graphs of levofloxacin, metronidazole benzoate and sulfamethoxazole spiked in aqueous buffer solution (a), urine (b) and plasma (c). Graphs were constructed using the calibration curve method in (a) and the standard addition method in (b) and (c). Normalized Area $= \frac{\text{Area of drug selected signal}}{\text{Area of the internal standard signal}} \times$ Amount of intternal standard in mg.

4. Conclusion

Selective, rapid, accurate and reproducible qHNMR method for the determination of levofloxacin, metronidazole benzoate and sulfamethoxazole in aqueous solutions was developed and validated. The method was successfully applied to the determination of individual drugs and their admixtures in pharmaceutical, urine and plasma samples. Linear ranges of 0.50–68.00, 0.13–11.30 and 0.24–21.00 mg per 0.6 mL solution were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole, respectively. Average recoveries % of 98.50 ± 1.82, 97.50 ± 2.21 and 98.50 ± 2.11 were obtained for the pure drugs in aqueous buffer solution, respectively. Average recoveries % of 98.50 ± 1.59, 99.20 ± 1.63 and 99.20 ± 1.92 were obtained in pharmaceutical, plasma and urine samples and its admixtures. Inter- and intra-day analyses gave average recovery % of 97.40 ± 2.26 and 99.40 ± 1.73, respectively.

Method LOD of 0.134, 0.045 and 0.064 and method LOQ of 0.446, 0.114 and 0.213 mg were obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole dissolved in aqueous borate or HCl solutions, respectively. In urine, method LOD of 0.115, 0.073 and 0.110 and method LOQ of 0.05, 0.245 and 0.366 mg were obtained, respectively. Corresponding values in plasma gave LOD of 0.153, 0.069 and 0.114 and LOQ of 0.250, 0.230 and 0.380 mg, respectively.

Instrument detection limits of 0.022, 0.018, and 0.020 mg per 0.6 mL were respectively obtained for levofloxacin, metronidazole benzoate and sulfamethoxazole in aqueous buffer solutions.

Developed method has demonstrated slight interference from pharmaceutical, plasma and urine matrices on selected signals. Chemical shifts of some signals were found sensitive to changes in the probe's temperature

Student *t*-test applied for the analytical results of drugs in pure, pharmaceutical, plasma, urine and their admixtures revealed an insignificant bias between the real and measured contents at 95% confidence level. Statistical *F*-test revealed insignificant differences in precisions between the developed qHNMR method and arbitrary selected reported HPLC methods used for analyzing these drugs in the same matrices.

Quantitative ^{1}H-NMR has shown to surpass chromatography in speed, precision, accuracy, and no needed for derivatization and reference standards. qHNMR also outdoes mass spectrometry due to its simplicity and separation is not required.

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The authors wish to dedicate this work to the spirit of Prof. B.N. Barsoum, chemistry department, Cairo University, Egypt, who passed away at the end of 2010 and was a member of this work team.

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